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Exploring the Therapeutic Potential of *Iphiona aucheri*: Modulation of Nitric Oxide Levels in RAW 264.7 Macrophages and Cytotoxic Effects on MCF-7 Human Breast Cancer and HT1080 Human Fibrosarcoma Cells

Azadeh Hamedi [1,2,3], Hamed Paslari [1, 4], Negar Azarpira [5], Reza Heidari [6], Leila Bordbar [1,2] and Ardalan Pasdaran [1,2,3] *

[1] *Department of Pharmacognosy, School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran*
[2] *Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
[3] *Phytopharmaceutical Technology and Traditional Medicine Incubator, Shiraz University of Medical Sciences, Shiraz, Iran*
[4] *School of Pharmacy, Shiraz University of Medical Sciences, Shiraz, Iran*
[5] *Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
[6] *Pharmaceutical Sciences Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*

*Corresponding Author: Email: pasdaran@sums.ac.ir

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ABSTRACT

The objective of this study was to examine phytochemical compounds and the cellular effects of the essential oil of *Iphiona aucheri*. The essential oil of the dried aerial parts of the plant with the recovery rate of 0.1% (v/w) was extracted through hydrodistillation by a Clavenger apparatus, and the chemical compounds were identified based on gas chromatography-mass spectrometry. The anti-inflammatory and cytotoxic effects of the essential oil were examined. The Griess assay was used to test the anti-inflammatory activity on the RAW 264.7 macrophage cell line. The MTT assay was used to test the cell viability on the RAW 264.7 macrophage, the HT1080 fibrosarcoma, and the MCF-7 cell lines. The essential oil of this plant consists of sesquiterpenes and monoterpenes, with more than 60% of it made up of compounds such as limonene, α-farnesene, and *trans*-caryophyllene. The essential oil demonstrated its anti-inflammatory effect on macrophage cells (RAW 264.7) by reducing nitric oxide production at concentrations below 100 µg/mL. Increasing the cell viability percentage of macrophage cells along with reducing the NO release rate at a concentration of 6.25 µg/mL suggested promising anti-inflammatory effects. The essential oil was cytotoxic to the human fibrosarcoma (HT1080) with an IC_{50} of 47.94±1 µg/mL.

Keywords: *Iphiona aucheri*, Anti-inflammatory, Nitric oxide, Cell viability

INTRODUCTION

The Asteraceae family comprises a significant number of flowering plants, encompassing about 1,600 genera and over 23,000 species [1]. The *Iphiona aucheri* plant is a member of the Asteraceae that grows in Iran, Saudi Arabia, Oman, Pakistan, and North East Africa [2]. In ethnomedicine, all parts of this plant, especially its aerial parts, have been used for cases such as pain [3], stomach ulcers, and snake and scorpion bites [4]. After heavy rain in the United Arab Emirates, this plant grew massively and was considered the cause of the death of some racing camels. In the studies conducted on different extracts of this plant, it was found that the petroleum, ethanol, and methanol extracts of the plant were not toxic to mice, while the aqueous extract of the plant caused the death of mice with hepatotoxicity [5]. Also, in another study, the administration of the plant to sheep caused the death of the sheep due to hemorrhagic liver cell necrosis [6]. In various studies conducted on the plant, various effects have been reported, including antioxidant effects, lowering blood sugar, cytotoxic effects, hepatoprotective effects [2], antibacterial effects [7], and anti-inflammatory effects [8]. Shah et al. observed the presence of proteins and amino acids, carbohydrates, glycosidic substances, alkaloids, saponins, and flavonoids in the total methanol extract and its fractions from the aerial portions of *I. aucheri* [9]. Moreover, the presence of anthraquinone, phlobatannin, tannin, terpene, saponin, and cardiac glycoside compounds in the total methanolic extract of this plant [8]. Isotussilagine, a type of pyrrolizidine alkaloid, and two diterpene glycosides,

carboxyatractyloside and atractyloside, have been isolated from *I. aucheri* [5]. Based on the investigation on the essential oil compounds of the aerial parts of *I. aucheri* by GC-MS, the essential oil of this plant mostly contained sesquiterpenes, and himachalol (41.9%) was the predominant compound [10].
This study aimed to investigate the therapeutic potential of *I. aucheri*, including the modulation of nitric oxide levels in RAW 264.7 macrophages and its cytotoxic effects on MCF-7 human breast cancer and HT1080 human fibrosarcoma cells.

MATERIAL AND METHODS

Plant Material

The aerial parts of *I. aucheri* were gathered from Bastak in March 2023. The plant was authenticated by a pharmacognosist, and a voucher specimen was held at the herbarium of the Medicinal Plants Processing Research Center, Shiraz University of Medical Sciences, for further reference.

Essential oil Extraction

The collected aerial parts of *I. aucheri* were dried at ambient temperature in the shade. Subsequently, it was transformed into a fine powder by a mill. Eight hundred grammes of the powder were subjected to hydro distillation for three hours using a glass Clevenger apparatus. Finally, anhydrous sodium phosphate (Na_2SO_4) was added to dehydrate the sample. The obtained essential oil was kept in a freezer at -8°C for further analysis.

Gas Chromatography-Mass Spectrometry

GC-MS (Agilent Technologies 7890 gas chromatograph) with stationary phase HP-5MS (Agilent Technologies 19091 S-433., 30 × 0.25 mm inner diameter) was used to identify the chemical compounds of the essential oil of *I. aucheri*. The mass spectrometer utilized was the Agilent Technologies model 5975 C, operating in electron impact mode at 70 eV. The temperature program started at 60°C and reached 220°C at a rate of 5°C/min and was kept at this temperature for 10 minutes. The flow rate for the carrier gas (helium) was 1 mL/min. The interface temperature of 280°C and mass range of 30-600 m/z were selected. The volatile compounds were identified via the NIST (National Institute of Standards and Technology) and Wiley libraries, prior publications, and by comparing retention times as well as mass spectra of reference compounds [11-14].

Evaluation of Cytotoxicity

Human breast cancer (MCF-7), human fibrosarcoma (HT1080), and macrophage (RAW 264.7) cell lines were used to evaluate the cytotoxic effects of essential oil of *I. aucheri* by the dimethylthiazol diphenyl tetrazolium bromide (MTT) method. The cell lines were cultivated in DMEM/F12 and RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 100 IU/mL penicillin G. After 24 hours of incubation (37°C temperature, 95% humidity, and 5% CO_2), the medium on the cells was drained and 1 mL of trypsin was added to the contents of the flask. In the next step, 3 mL of culture medium was added and then centrifuged (5 min., 1500 rpm). The supernatant was removed and fresh culture medium was added to the falcon tube to make the cell pellet a suspension. The number of cells in 1 mL of cell suspension was obtained using the trypan blue dye. For the cultivation of MCF-7 and HT1080 cell lines, 7000 cells were cultured in each well of a 96-well plate. For macrophage (RAW 264.7) 6000 cells were cultured in each well. The plate was placed in an incubator with a temperature of 37°C, 95% humidity, and 5% CO_2.
Following a 24-hour period, the cells were subjected to various concentrations (6.25, 12.5, 25, 50, 100, and 200 µg/mL) of the essential oil. In the cytotoxicity assay, paclitaxel served as a positive control, whilst blank wells and wells containing cells with only medium were designated as untreated negative controls. After 48 hours of incubation, the medium on the wells was drained and MTT solution (0.5%, in phosphate buffer pH=7.2) was added to them. After 4 hours of incubation, the plate was centrifuged for 5 minutes at 1500 rpm. The wells were drained and 100 microliters of DMSO were added to each well. Finally, the absorption of the wells was measured at 570 and 630 nm by an enzyme-linked immunosorbent assay (ELISA) plate reader (BioTek Instruments®/USA). All experiments were performed in three replicates.

Nitric Oxide Assay

To investigate the anti-inflammatory effects of the *I. aucheri* essential oil on the macrophage cells, the nitric oxide assay was applied. 150,000 macrophage cells were cultured as described above. Cells were subjected to various concentrations (2, 6.25, 12.5, 25, 50, 100, and 200 µg/mL) of the essential oil and lipopolysaccharide (LPS) (1 µg/mL). After 24 hours, 100 microliters of the well supernatant of the macrophage cell line (RAW 264.7) were placed in a new 96-well plate for the NO test. 100 microliters of Griesse solution (0.5% sulfanilamide, 0.05% of n-1-naphthylethylene diamine dihydrochloride, 2.5% phosphoric acid) was added to each well, and 20 minutes later, the absorption at 570 nm was measured by the Elisa reader. The cells that received only LPS were considered as the control.

Statistical Analysis

The experiments were conducted in three replicates. The findings are reported as mean ± standard deviation (SD). Statistical analysis was conducted utilising Prism 8.0.2 (Graphpad Software Inc.).

The Dunnett's multiple comparisons test subsequent to ANOVA was employed. All results were evaluated based on statistical significance with p-values below 0.05.

RESULTS

α-farnesene

In the present study, the essential oil of the *I. aucheri* plant was obtained by the hydrodistillation method with a recovery rate of 0.1% (v/w). The phytochemicals in the essential oil of *I. aucheri* were identified using the gas chromatography-mass spectrometry (GC-MS) technique and the identified compounds are listed in Table 1. In this study, 39 compounds that constitute 90.19% of the essential oil were recognized. The dominant compounds were limonene (24.72%), α-farnesene (8.52%), and trans-caryophyllene (7.03%). The identified compounds are mostly sesquiterpenes (39.4%) and monoterpenes (41.01%). The essential oil was cytotoxic to the human fibrosarcoma (HT1080) with an IC_{50} of 47.94±1 µg/mL. Conversely, although it decreased the cell viability of the MCF-7 cells, the IC_{50} value (100 µg/mL ≤IC_{50}), this cytotoxicity was not significant on MCF-7 cell lines (data not shown). Moreover, the essential oil of *I. aucheri* at low concentration (6.25 µg/mL) increased the cell viability of macrophages (RAW 264.7 cell line) that activated with LPS but at higher concertation, it decreased the cell viability with the IC_{50} which was calculated 36.90±3 µg/mL (Figure 1). To evaluate the anti-inflammatory effects of the *I. aucheri* essential oil, the release of nitric oxide in the macrophages (RAW 264.7 cell line) in the presence of LPS was evaluated. RAW 264.7 macrophage cells have demonstrated activation in response to LPS and subsequent release of nitric oxide (NO), rendering them an optimal model for investigating inflammation [15]. In this study, the anti-inflammatory effect of the essential oil extracted from the *I. aucheri* plant on RAW 264.7 macrophage cells were investigated by the Griesse test, and its cytotoxic effect on RAW 264.7 macrophage cells, fibrosarcoma and breast cancer was tested by the MTT method. At concentrations less than 100 µg/mL, a decrease in nitric oxide release was observed compared to the control negative group, but this decrease was only significant at low concentrations (less than 6.25 µg/mL). By increasing the concentration of the essential oil, the cell viability percentage of macrophage cells decreased and IC_{50} was estimated as 36.90 ± 3 µg/mL. The essential oil of the *I. aucheri* on fibrosarcoma and breast cancer cell lines decreased the cell viability percentage with increase in the concentrations, and the essential oil was more toxic on the fibrosarcoma cell line; the cell viability percentage of fibrosarcoma cell line at the concentration of 50 µg/mL of the essential oil was 45.57% and the IC_{50} was estimated as 47.94 ± 1 µg/mL. As seen in Figure 1, at low concentrations (6.25 and 3.1 µg/mL) the release of NO was significantly decreased ($p < 0.05$). At the concentration of 200 µg/mL of the essential oil, the release of NO increased, which seems to be due to the toxicity of the essential oil on the macrophage cell line based on Figure 1.

Table 1 The identified compounds in the essential oil of *I. aucheri.*

Component	Retention Time	Area %	Calculated KI	Reference KI	classification
β-Myrcene	5.83	0.59	991.18	992	Acyclic unsubstituted monoterpene
Limonene	6.81	24.72	1034.10	1035	Monoterpene
cis-Ocimene	6.88	0.40	1037.26	1037	Monoterpene
β-Ocimene	7.14	2.23	1048.32	1046	Monoterpene
Linalool	8.40	1.17	1101.15	1104	Noncyclic monoterpenoid
Decanal	11.14	0.35	1206.20	1200	Aldehyde
Nerol	11.74	0.72	1229.02	1232	Monoterpenoid
Z-Citral	12.11	0.66	1243.02	1249	Acyclic monoterpene aldehyde
Geraniol	12.44	0.87	1255.65	1258	Monoterpenoid
E-Citral	12.89	0.84	1272.73	1273.	Acyclic monoterpene aldehyde
2,4-Decadienal	14.06	0.40	1317.66	1320	Polyunsaturated fatty aldehyde
δ-Elemene	14.67	1.45	1341.48	1340	Sesquiterpenes
Citronellyl acetate	15.01	0.97	1354.40	1354	Monoterpenoid
Neryl acetate	15.32	2.30	1366.42	1365	Terpenoid
Geranyl acetate	15.85	3.68	1387.08	1392	Monoterpene
β-Damascenone	15.90	1.19	1389.34	1386	Rose ketone
β-Elemene	16.11	2.49	1397.28	1394	Sesquiterpenes
trans-Caryophyllene	16.86	7.03	1427.67	1423	Bicyclic sesquiterpene
2-Norpinene, 2,6-dimethyl-6-(4-methyl-3-pentenyl)-	17.17	1.00	1440.20	1433	Sesquiterpene
α-Humulene	17.66	1.12	1460.08	1452	Monocyclic sesquiterpene
Germacrene-D	18.33	1.82	1487.26	1480	Sesquiterpene
β-Selinene	18.47	0.73	1492.94	1489	Sesquiterpene
α-selinene	18.67	0.57	1501.02	1494	Sesquiterpene
α-Farnesene	18.94	8.52	1512.69	1507	Sesquiterpene
γ-Cadinene	19.12	0.34	1520.14	1524	Bicyclic sesquiterpene
δ-Cadinene	19.33	0.49	1529.29	1528	Bicyclic sesquiterpene
Caryophyllene oxide	20.05	4.30	1560.07	1561	Sesquiterpenoid oxide
Germacrene B	20.19	6.43	1565.77	1562	Sesquiterpene
Geranyl isovalerate	21.25	1.86	1611.48	1604	Monoterpenoid
α-Caryophylladienol	21.98	0.30	1644.26	1661	Bicyclic sesquiterpene
Cyclotetradecane	22.72	0.96	1677.36	1673	Cyclic alkane
Heptadecane	23.21	0.25	288.43	282.99	Alkane
Pentadecanal	23.57	0.87	1715.89	1715	Alpha-hydrogen aldehyde
trans-Farnesol	23.76	0.77	1724.92	1722	Acyclic sesquiterpene alcohol
Tetradecanoic acid	24.62	0.67	1765.21	1765	Saturated fatty acid
Hexahydrofarnesyl acetone	26.33	2.04	1847.89	1838	Sesquiterpene
Pentadecanoic acid	26.62	0.34	1861.90	1869	Straight-chain saturated fatty acid
Farnesyl acetone	27.81	0.39	1921.33	1927	Sesquiterpene acetone
Hexadecanoic acid	28.76	4.36	1970.58	1964	Saturated long-chain fatty acid

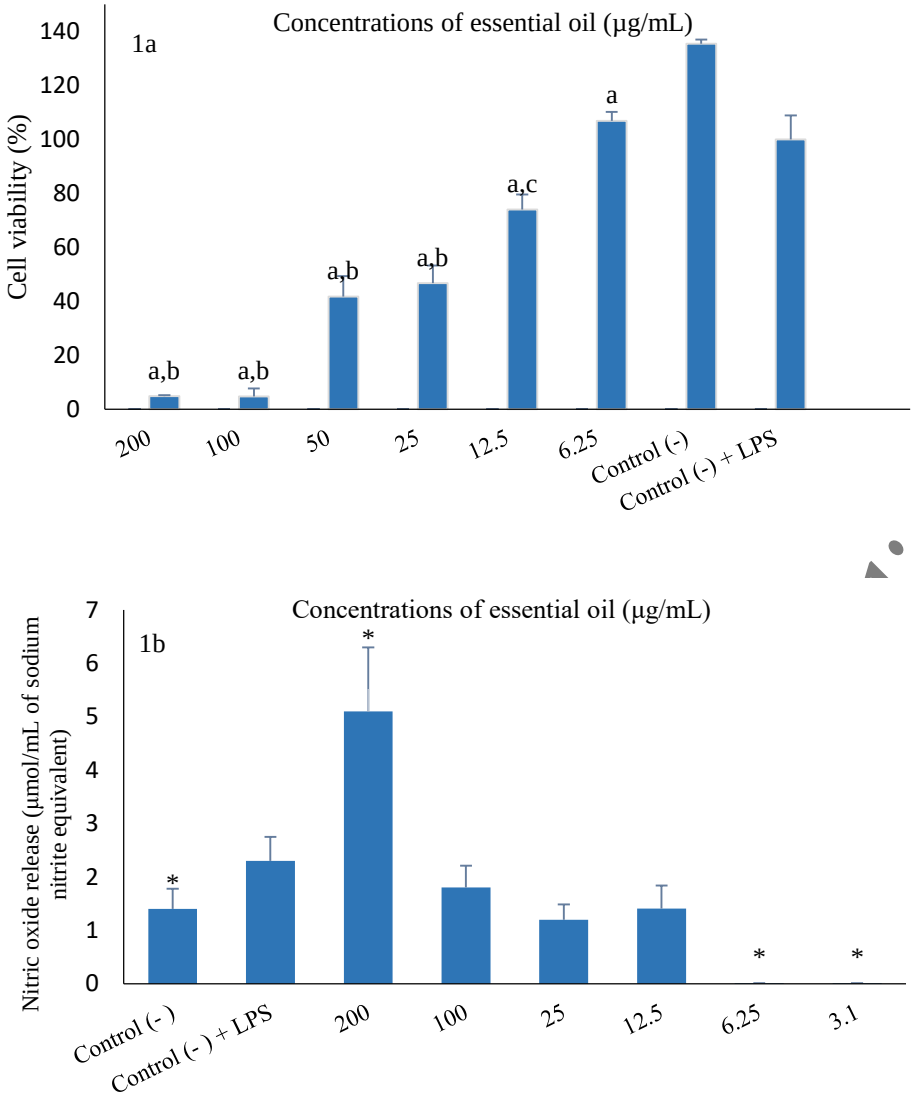

Fig. 1 Cell viability of macrophage cell line treated with different concentrations of essential oil of *I. aucheri* (1a) and the results of NO assay presented as released from RAW264.7 macrophage cells as a result of LPS stimulation and cell treatment with different concentrations of essential oil (1b). Control (-): Untreated macrophage cell; Control (-) + LPS: Untreated macrophage cell + LPS). Data were reported as SD ± Mean. a= significantly different with control (-) group ($p < 0.0001$); b= significantly different with control (-) + LPS group ($p < 0.0001$); c= significantly different with control (-) + LPS group ($p < 0.005$).

DISCUSSION

Inducible nitric oxide synthase (iNOS) generates nitric oxide (NO) in response to inflammation, which leads to inflammatory responses, including increased vascular permeability, edema, and the synthesis of inflammatory mediators, therefore exacerbating inflammation. Research indicates that iNOS is chiefly implicated in the inflammatory response and is expressed following activation with LPS [16]. This molecule (LPS) stimulates innate immune cells, including macrophages and neutrophils, which produce proinflammatory mediators such as IL-1 and TNF-α, as well as free radicals. This leads to a notable inflammatory response. Nitric oxide (NO) can induce oxidative damage [17]. Therefore, some natural antioxidants including the molecules that are identified in the essential oil of *I. aucheri* are supposed to decrease these inflammatory mediators. The results of GC-MS analysis in the present work showed that most of the compounds in the essential oil obtained from the aerial parts of I. aucheri are sesquiterpene and monoterpene compounds. More than 60% of essential oil components of the I. aucheri are limonene (24.72%), α-farnesene (8.52%), trans-caryophyllene (7.03%), germacrene B (6.43%), hexadecanoic acid (4.36%), caryophyllene oxide (4.30%), geranyl acetate (3.68%) and β-elemene (2.49%) respectively. In a report by Rostaian et al., the essential oil of the aerial parts of I. aucheri collected from Kerman

mainly contained sesquiterpene compounds, and the dominant compounds of the essential oil were himachalol (41.9%), β-himachalene (18.6%), and bornyl acetate (15.5%) [10]. In a study conducted on the ethanolic extract of I. aucheri collected from Saravan, Sistan and Baluchistan province, by GC-MS by Sharifirad et al., three dominant compounds of the extract were identified as hemachalol, β-himachalene, and bornyl acetate, respectively [18]. Variations in the phytochemical of the plants might be due to the climate variations and soil conditions, harvesting seasons, geographical origin, and procedures involved in the extraction. Seasonal changes and weather conditions are the major factors affecting the phytoconstituents [19].

Terpenes are a class of secondary metabolites present in natural sources such as plants. Some terpenes exhibit anti-inflammatory effects. Caryophyllene is a type of sesquiterpene found in black pepper, cloves, and cannabis. By binding to the CB2 receptor, this compound reduces the production of pro-inflammatory cytokines and inhibits the inflammation pathway [20]. Limonene is a monoterpene compound found in citrus. In a study conducted on ulcerative colitis rats, limonene reduced the expression levels of MMP-2 and 9-mRNA through regulation of iNOS, COX-2, PGE2, TGF-β, and ERK1/2 signaling pathways, indicating antioxidant and anti-inflammatory effects [21]. A study on the volatile oil of *Gardenia jasminoides* revealed that farnesene (a sesquiterpene) is its prominent component. On the MH-S cell line, farnesene decreased the release of NO from the cell in a dose-dependent manner [22]. Since these three compounds constitute more than 40% of the essential compounds of *I. aucheri* in this study, the anti-inflammatory effect of the essential oil can be attributed to the presence of these compounds and other terpene compounds in the essential oil.

CONCLUSION

The study investigated the phytoconstituents and biological effects of the essential oil extracted from the aerial parts of *I. aucheri*. More than 40% of essential oil included limonene, α-farnesene, and *trans*-caryophyllene. In contrast to previous reports, himachalol was not detected in the essential oil.

The *I. aucheri* essential oil in concentrations less than 100 µg/mL reduced nitric oxide production. Increasing the cell viability percentage of macrophage cells along with reducing the NO release rate at a concentration of 6.25 µg/mL suggested promising anti-inflammatory effects. The essential oil was cytotoxic against fibrosarcoma cells.

Conflicts of Interest

The authors declared no potential conflicts of interest.

Authors' contributions

All authors contributed in this work equally.

Acknowledgment

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Ethical Considerations

This research was conducted *in vitro* and did not include any human or animal subjects. The authors examined ethical considerations in research within the study. The study received approval from the ethical committee of Shiraz University of Medical Sciences (IR.SUMS.REC.1402.404).

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